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Activation of signal transducer and activator of transcription 5 (STAT5) is linked to β1-integrin protein abundance in unilaterally milked bovine mammary glands

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ABSTRACT

Prolactin (PRL) is important in the regulation of milk synthesis in mammary epithelial cells (MEC). In cattle, circulating levels of PRL are not limiting, suggesting the possible involvement of other factors that may control the response to PRL at the cellular level. The effects of milking frequency (MF) on milk synthesis are controlled locally within mammary glands and involve PRL signaling. To further investigate this relationship between MF and PRL signaling, udder halves of 17 dairy cows were milked either 4 times a day (4×) or once a day (1×) for 14 d in early lactation. Mammary biopsies were obtained 3 to 5 h following milking from both udder halves of 10 cows, and changes in PRL and associated pathways were measured. The abundance of *STAT5A* mRNA was higher after 4× milking, whereas that of the PRL receptor (*PRLR*) and *STAT3* were lower relative to that after 1× milking. In 4× mammary tissues, the protein levels of STAT5, activated STAT5, and β1-integrin were higher, whereas the those of the long isoform of PRL receptor and activated STAT3 were lower than 1× tissues. The activation of STAT5 correlated strongly with major milk protein mRNA abundance (r = 0.86 to 0.94) and β1-integrin protein levels (r = 0.91). These results confirm that major milk protein gene expression is associated with STAT5 activation and suggests that the STAT5 and β1-integrin signaling pathways are linked. Modulation of β1-integrin abundance in response to changes in MF may be a mechanism that controls the MEC ability to respond to PRL and therefore its secretory activity.

Key words: milking frequency, mammary, milk synthesis, cell signaling

INTRODUCTION

In lactating mammary glands, prolactin (**PRL**) is important for the regulation of milk synthesis in mammary epithelial cells (**MEC**; Flint and Knight, 1997). The PRL signal is transmitted into the cell through its receptor (**PRLR**), which facilitates the activation of 2 closely related signaling proteins, signal transducer and activator of transcription (**STAT**)5A and 5B. When activated, the STAT5 proteins form homodimers and are transported to the nucleus, where they activate expression of genes involved in milk synthesis. This relationship between PRL signaling and milk synthesis has been well established in rodent models (reviewed in Flint and Knight, 1997). Administration of bromocriptine, which blocks PRL release from the pituitary gland, inhibits further milk secretion in rodents (Shaar and Clemens, 1972; Knight et al., 1986); however, the relationship is unclear in cattle. During lactogenesis, in the period leading up to parturition, there is a surge in circulating PRL (Ingalls et al., 1973). Inhibition of this surge with bromocriptine can decrease subsequent milk yield (**MY**; Akers et al., 1981). However, once lactation has been established, blocking release of PRL with bromocriptine has little effect on MY (Smith et al., 1974). Nevertheless, recent studies have shown that another PRL release-blocking agent, quinagolide, applied over a longer period in mid-lactation does decrease MY (Lacasse et al., 2011), suggesting that PRL may have a galactopoietic effect under certain conditions.

Unilateral MF (**UMF**) models, where udder halves are milked independently at differing MF, have demonstrated that the effects on MY and MEC activity are predominantly controlled locally within the treated mammary gland (Stelwagen and Knight, 1997; Wall and McFadden, 2007; Murney et al., 2015). Dairy cows are commonly milked twice a day (**2×**), but increasing MF to 4 times a day (**4×**) can increase MY by up to 20% compared with 2× (Stelwagen, 2001). This response is immediate (Hillerton et al., 1990; Murney et al., 2015) and has been observed at most stages of

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lactation (reviewed in Erdman and Varner, 1995). Similarly, decreasing MF from 2× to once a day (**1×**) has a negative effect on MY (Carruthers et al., 1993; Rémond et al., 1999).

The mechanisms underlying the MY response to MF are not well understood, but are most likely a function of secretory MEC number, activity, or both. In mammary glands during lactation, the number of MEC is controlled by the rates of proliferation and apoptosis (Capuco et al., 2001), which may be modulated by MF (Boutinaud et al., 2013; Murney et al., 2015). However, changes in cell number are thought to be incremental and occur over the course of days to weeks (Capuco et al., 2001) and are unlikely to account for the 60% shift in MY in response to differential MF that occurs within 2 d of commencement of treatment (Murney et al., 2015). Therefore, at least part of the response to MF may be through the modulation of the secretory activity of MEC. An increase in the mRNA abundance of both the long and short isoforms of the PRLR (Bernier-Dodier et al., 2010) occurs in response to increased MF. In addition, quinagolide treatment had more of an effect on the MY of udder halves milked 2× compared with 1× (Lacasse et al., 2011). These results suggest a possible link between MF and PRL signaling that is manifested through alteration of the sensitivity of MEC to circulating PRL.

Signaling from the extracellular matrix via integrins is necessary for appropriate MEC differentiation and milk synthesis (Sympson et al., 1994; Roskelley et al., 1995). It has been demonstrated in mouse knockout models that β1-integrin is essential for mammary gland development and sustained STAT5 activation (Naylor et al., 2005; Akhtar and Streuli, 2006). In normal mammary glands of mice (McMahon et al., 2004) and cows (Singh et al., 2008), the level of β1-integrin protein decreases during involution, which suggests that the abundance of this protein is modulated during physiological changes in mammary tissue function. Furthermore, in cow mammary glands, this decrease coincides with a decrease in STAT5 activation (Singh et al., 2009).

The ability of MEC to respond to PRL can be perturbed by the presence of antagonistic factors. In mammary glands, STAT3 activation is pro-apoptotic and can be induced by the leukemia inhibitory factor (LIF) pathway (Kritikou et al., 2003). Activation of STAT3 has been shown to occur during involution in both mouse and bovine mammary glands (Kritikou et al., 2003; Singh et al., 2009) and coincides with the inactivation of STAT5. Evidence from in vitro models suggests that the activation of STAT3 can inhibit activation of STAT5 and vice versa (Clarkson et al., 2006; Granillo et al., 2007), but whether this occurs in vivo is unclear.

In this study, a UMF model was used to investigate PRL signaling in mammary glands of dairy cows. We hypothesized that the MY response to increased MF is a result of an increase in MEC sensitivity to circulating PRL. If so, this could be manifested through changes in the abundance of constituents of the PRL signaling pathway or cross talk from other stimuli, such as extracellular matrix interactions via β1-integrin or STAT3 activation. We therefore investigated changes in these signaling proteins, and their encoding mRNA, in response to altered MF.

MATERIALS AND METHODS

Animals and Treatments

All animal manipulations were conducted in compliance with the rules and guidelines of the Ruakura Animal Ethics Committee. Animal management and treatments have been described previously (Murney et al., 2015). Briefly, the udder halves of 17 cows were randomly assigned a MF of either 4× or 1× (4× in one udder half at 0500, 1100, 1700, and 2300 h, and 1× in the other udder half at 1100 h) for 14 d from 5 ± 2 DIM. The MY of 4× and 1× udder halves were 8.4 ± 0.5 kg/d and 8.5 ± 0.5 kg/d, respectively, before commencement of the treatment, and were 15.0 ± 0.7 kg/d and 7.5 ± 0.3 kg/d for 4× and 1× treatments, respectively, by the end of the 14-d period. Biopsies of mammary gland tissue were taken from both rear quarters of 10 cows 3 to 5 h after the 1100 h milking, as previously described (Farr et al., 1996). Briefly, this involved excision of approximately 0.5 g of alveolar tissue from a site distal to the cistern and major ducts using a modified purpose-built drill rotating stainless steel cannula. A portion of the mammary tissue was fixed overnight in 4% paraformaldehyde and processed into wax as described previously (Singh et al., 2005). The remainder was snap-frozen in liquid nitrogen for subsequent molecular analysis of mRNA and protein.

RNA Isolation and Reverse Transcription

Total RNA was isolated from 100 mg of mammary tissue using Trizol reagent (Life Technologies, Grand Island, NY) according to the manufacturer's instructions and quantified using a Nanodrop 1000 spectrophotometer (Nanodrop, Wilmington, DE). The RNA integrity was measured on an Agilent 2100 Bioanalyser (Agilent, Santa Clara, CA) with an RNA integrity number >5 considered sufficient for real-time, reverse transcription-PCR analysis. For cDNA synthesis, 1 μg of total RNA was purified using RNAeasy columns (Qiagen, Valencia, CA), according to the supplied RNA clean-up protocol